DISCOVERY

Prevention of the NMDA glutamate receptor antagonist ketamine-induced oxidative stress, brain neuronal degeneration and liver injury by methylene blue

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ABSTRACT

Methylene blue (MethyB) was shown to possess potential neuro- and liver protective properties. The aim of this study was to investigate the effect of MethyB on brain neurodegeneration and liver injury induced by ketamine. Rats were treated with single intraperitoneal (i.p.) injection of ketamine (35 mg/kg) either alone or combined with MethyB at doses of 20 or 40 mg/kg and euthanized 4h later. The determination of biomarkers of oxidative stress including malondialdehyde, nitric oxide and reduced glutathione as well as paraoxonase-1 (PON-1) was carried out in brain and liver tissue. In addition, the concentrations of acetylcholinesterase (AChE) and Aβ-peptide were determined in brain tissue and histological studies were done using haematoxylin and eosin staining. Results indicated that compared to the saline control, ketamine-treated rats exhibited significantly elevated malondialdehyde, decreased nitric oxide levels, as well as depletion of reduced glutathione and decreased PON-1 activity in brain and liver. There were also significant decrements in brain Aβ-peptide, AChE levels by ketamine treatment. The presence of dark shrunken cortical neurons with deeply stained pyknotic nuclei and shrunken degenerated hippocampus pyramidal cells having dark eosinophilic cytoplasm with pyknotic nuclei was detected in brain after ketamine injection. The liver of these animals exhibited severe degeneration of hepatocytes and perivascular infiltrations of inflammatory cells with dilated sinusoids. In ketamine-treated rats, the administration of MethyB resulted in significant inhibition of lipid peroxidation, increased reduced glutathione levels and PON-1 activity, while it decreased nitric oxide in brain and liver. It had no effect on AChE but caused further decrease in Aβ-peptide in brain of ketamine-treated rats. MethyB conferred dose-dependent protection against the ketamine-induced histological changes in brain and liver with the higher dose bringing about almost normalization of these tissues. It is concluded that MethyB exerts a neuro- and hepato-protective effects against ketamine toxicity and these may involve anti-oxidative actions as a potential mechanism.

Keywords: Methylene blue, ketamine, neurotoxicity, hepatotoxicity, nitric oxide, amyloid Aβ-peptide, acetylcholinesterase.

1. INTRODUCTION

Ketamine is a phenylcyclidine hydrochloride derivative and a dissociative anesthetic with psychedelic properties (Wolff, 2012). It binds selectively to the phencyclidine (PCP) binding site in the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype on GABA-ergic interneurons. Ketamine acts as a non-competitive NMDA glutamate receptor antagonist with a resultant increase in the release of the excitatory neurotransmitter glutamate in the prefrontal cortex (Mion amd Villevieille, 2013). It is used at subanesthetic doses for pain or mild sedation in humans (Niesters et al., 2014; Ahern et al., 2015) and also as an adjunct to morphine to control moderate to severe acute pain (Beaudoin et al., 2014).

Ketamine induces schizophrenic-like states in both humans and rodents (Neill et al., 2010; Pomarol-Clotet et al., 2006) and has thus been utilized as an animal model of schizophrenia (Wilson and Koenig, 2014). The drug has rapid-acting and long-lasting antidepressant effects; and because of its dissociative properties, is popular as a drug of abuse both among young people in clubs, large music parties and raves and among spiritual seekers (Lankenau and Clatts, 2004; Trujillo et al., 2011). Free radical mediated oxidative damage has been implicated in the development of several neurodegenerative and neurological disorders (Klein and Ackerman, 2003).

Oxidative stress ensues when oxidant free radicals are produced at amounts that exceed the antioxidant capacity of the cell with consequent oxidative damage to membrane lipids, enzyme proteins, and nucleic acid leading to cellular instability or even cell death (Sies, 1997). Reactive oxygen metabolites such as superoxide radical ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) are generated by the mitochondrial respiratory chain as byproducts of oxygen metabolism. The brain with its high metabolic demands and hence oxygen utilization produces increased amounts of mitochondrial superoxide.

Excitotoxic amino acids, and the autoxidizable neurotransmitters noradrenaline, dopamine and serotonin are other sources of free radicals. The oxidation of the catecholamines produces superoxide anion radical and hydrogen peroxide as well as quinines and semiquinones capable of binding to thiol groups and depleting reduced glutathione in the cell. Besides, the brain is rich in polyunsaturated fatty acids which are the target for oxidant species. These factors coupled with insufficient antioxidants, particularly catalase levels are believed to account for an increase in the vulnerability of the brain to increased levels of oxidative stress (Halliwell, 2006; Halliwell, 2009).

When administered to rodents, ketamine caused a significant increase in brain lipid peroxidation while decreasing reduced glutathione concentrations. It also increased interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) and induced neuronal degeneration, apoptosis and perineuronal vacuolation in the cerebral cortex and striatum (Abdel-Salam et al., 2021). Methylene blue (MethyB) is a synthetic phenothiazine dye that, in the last years, has gained interest, being a potential candidate for the treatment of central nervous system pathologies.

MethyB has been shown to protect against neuronal damage in models of ischaemia/reperfusion injury (Bardakci et al., 2006), Huntington's disease (Sontag et al., 2012), Parkinson's disease (Abdel-Salam et al., 2014), amyotrophic lateral sclerosis (Dibaj et al., 2010), and traumatic brain injury (Talley-Watts et al., 2014). It also prevented brain neuronal death induced by such toxicants as Malathion (Abdel-Salam et al., 2016a) or toluene (Abdel-Salam et al., 2016b) in rats. In this study, we investigated the potential preventive effects of MethyB on ketamine-induced injury of brain and liver in the rat and possible underlying mechanisms using biochemical and histological approaches.

2. MATERIALS AND METHODS

Animals

This study was conducted on male Sprague–Dawley strain rats weighing 160–170 g of body weight. Rats were kept under standardized conditions and had free access to a standard laboratory chow and water. The animal studies were done according to regulations of the Institute Ethics Committee and Guide for Care and Use of Laboratory Animals of US National Institutes of Health (Publication No. 85-23, revised 1996). Six rats were used per group.

Chemicals and reagents

Methylene blue was purchased from Sigma (St Louis, MO, USA). Ketamine was obtained from the Ministry of Justice (Egypt). Other chemical and reagents were obtained from Sigma (St Louis, USA). Methylene blue and ketamine were dissolved in isotonic

(0.9% NaCl) saline solution before use. The remaining of chemicals and reagents were of the analytical grade and purchased from Sigma (St Louis, MO, USA). The doses of ketamine and methylene blue used were chosen based on previous studies (Abdel-Salam et al., 2016a; Abdel-Salam et al., 2016b; Abdel-Salam et al., 2021).

Study design

The following groups were used: Group 1 (normal control) received i.p. saline (0.2 ml/rat). Groups 2, 3 and 4 were i.p. treated with ketamine at a dose of 35 mg/kg. Thereafter, group 2 received i.p. saline and kept as a positive control. Meanwhile, groups 3 and 4 were given MethyB blue at doses of 20 or 40 mg/kg. Rats were euthanized after 4h by decapitation under light ether anesthesia, their brains, and livers quickly removed, washed with ice-cold 0.9% NaCl solution, weighed, and then stored at -80°C. The tissues were homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 10% w/v for the biochemical assays. For the histological studies, representative brain and liver samples were kept in 10% neutral buffered formalin.

Biochemical analyses

Determination of lipid peroxidation

Malondialdehyde, an end product of lipid peroxidation was determined by measuring thiobarbituric reactive substances (TBAS) using the method by Nair and Turner, (1984) in which TBAS react with thiobarbituric acid forming TBA-MDA adduct and the absorbance is read at 532 nm using spectrophotometer.

Determination of nitric oxide

Nitric oxide estimated as nitrate/nitrite was determined by the use of Griess reagent. In this assay, nitrate is converted to nitrite by nitrate reductase. The Griess reagent then reacts with nitrite forming a deep purple azo compound. The absorbance is read at 540 nm using a spectrophotometer (Archer, 1993).

Determination of reduced glutathione

Reduced glutathione (GSH) was determined using Ellman´s reagent (DTNB (5, 5′-dithiobis (2-nitrobenzoic acid))) which is reduced by the free sulfhydryl group on the GSH molecule generating 5-thio-2-nitrobenzoic acid. The latter has yellow color and can be determined by reading absorbance at 412 nm (Ellman, 1959).

Determination of paraoxonase-1

The arylesterase activity of PON-1 was determined by a colorimetric method which uses phenyl acetate as a substrate. In this assay, PON-1 catalyzes the cleavage of phenyl acetate resulting in the formation of phenol. The rate of phenol formation was measured via monitoring the increase in the absorbance at 270 nm at 25°C. One unit of arylesterase activity is equal to 1 μmole of phenol formed per minute. The PON1 activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 $M^{-1}cm^{-1}$ (Haagen and Brock, 1992).

Quantification of acetylcholinesterase

Acetylcholinesterase (AChE) concentration was determined in supernatants using an ELISA kit purchased from NOVA (Bioneovan Co., Ltd., Daxing Industry Zone, Beijing, China) according to the manufacturer's instructions.

Quantification of amyloid Aβ peptide

Rat amyloid beta peptide 1-41 (Aβ 1-42) ELISA Kit (SinoGeneClon Biotech Co., Ltd) was used according to the manufacturer's instructions.

Histological studies

Five μm thick paraffin sections were stained with haematoxlin and eosin (Drury and Walligton, 1980) and investigated by light microscope (Olympus Cx 41 with DP12 Olympous digital camera. Olympous optical Co. Ltd, Tokyo, Japan).

Statistical analyses

Data in the study were presented as mean ± SEM. Statistical significance was determined with the use of one-way ANOVA with Duncan's multiple range test. GraphPad Prism software, version 6 (GraphPad Prism Software Inc., San Diego, CA, USA) was used. A probability value of less than 0.05 was considered as statistically significant.

3. RESULTS

Effect of MethyB on ketamine-induced biochemical changes in brain

Brain lipid peroxidation

Rats given i.p. injection of ketamine exhibited significantly increased brain MDA level by 50.2% compared to the saline group (31.80 ± 1.12 *vs.* 21.17 ± 0.67 nmol/g tissue). The ketamine-treated rats receiving MethyB at 20 or 40 mg/kg exhibited significantly lower MDA values by 45.3% and 49.3% in comparison to the ketamine control (17.41 ± 1.38 and 16.13 ± 1.25 *vs*. 31.80 ± 1.12 nmol/g tissue) (Figure 1A).

Brain nitric oxide

Nitric oxide decreased by 16.5% (15.31 ± 0.31 *vs.* 18.34 ± 0.63 μmol/g tissue) by ketamine compared to the saline control value. Nitric oxide level was further and significantly decreased by 18% MethyB at 40 mg/kg compared to ketamine control (12.57 ± 0.44 *vs.*15.31 ± 0.31 μmol/g tissue) (Figure 1B).

Brain reduced glutathione

The level of GSH fell by 26.2% (2.51 ± 0.09 vs. 3.40 ± 0.11 μmol/g tissue) by ketamine compared to the saline control. Administration of MethyB at 20 or 40 mg/kg to ketamine-treated rats produced significantly higher levels of GSH than the ketamine control group (26.7% and 30.7% increments: 3.18 ± 0.11 and 3.28 ± 0.15 vs. 2.51 ± 0.09 μmol/g tissue) (Figure 1C).

Brain paraoxonase-1

Ketamine caused a significant decrease in brain PON-1 activity by 39.8% compared to the saline control group (6.81 ± 0.38 *vs*. 11.32 ± 0.33 kU/l). This effect of ketamine was reversed by treating rats with MethyB at 20 mg/kg with even MethyB at 40 mg/kg increasing PON-1 activity above that of the saline group (Figure 1D).

Brain acetylcholinesterase

Ketamine caused a significant decrease in brain AChE concentration by 55.6% compared with the saline control (1.62 ± 0.21 *vs*. 3.65 ± 0.19 ng/ml). MethyB given at 20 or 40 mg/kg had no significant effect on AChE in brain of rats treated with ketamine (1.66 ± 0.12 and 1.7 ± 0.08 *vs*. 1.62 ± 0.21 ng/ml) (Figure 2A).

Brain Aβ-peptide

The concentration of Aβ-peptide was markedly and significantly decreased by 57.8% in the brain of ketamine-treated rats (3.35 ± 0.11 *vs*. 7.94 ± 0.24 pg/ml). MethyB given to ketamine-treated rats resulted in further decrease in Aβ-peptide by 53.7% and 60.9% compared to the ketamine control value (1.55 ± 0.04 and 1.31 ± 0.19 *vs*. 3.35 ± 0.11 pg/ml) (Figure 2B).

Effect of MethyB on ketamine-induced biochemical changes in liver

Liver lipid peroxidation

Compared to the saline-treated group, ketamine injection caused a significant increase in the level of MDA by 65.7% (56.18 ± 2.24 *vs*. 33.90 ± 1.46 nmol/g tissue). Rats treated with ketamine and administered MethyB showed significantly lower liver MDA content by 35.5% and 43.1% compared to the ketamine control animals (36.22 ±1.36 and 31.98 ± 0.84 *vs*. 56.18 ± 2.24 nmol/g tissue) (Figure 3A).

Liver nitric oxide

No significant difference in nitric oxide was observed between ketamine treated group and saline control (21.79 ± 0.69 *vs*. 24.54 ± 0.89 μmol/g tissue). Meanwhile, ketamine-treated rats given MethyB 40 mg/kg exhibited significantly lower nitric oxide level by 14% compared to the ketamine control value (18.74 ± 0.68 *vs*. 21.79 ± 0.69 μmol/g tissue) (Figure 3B).

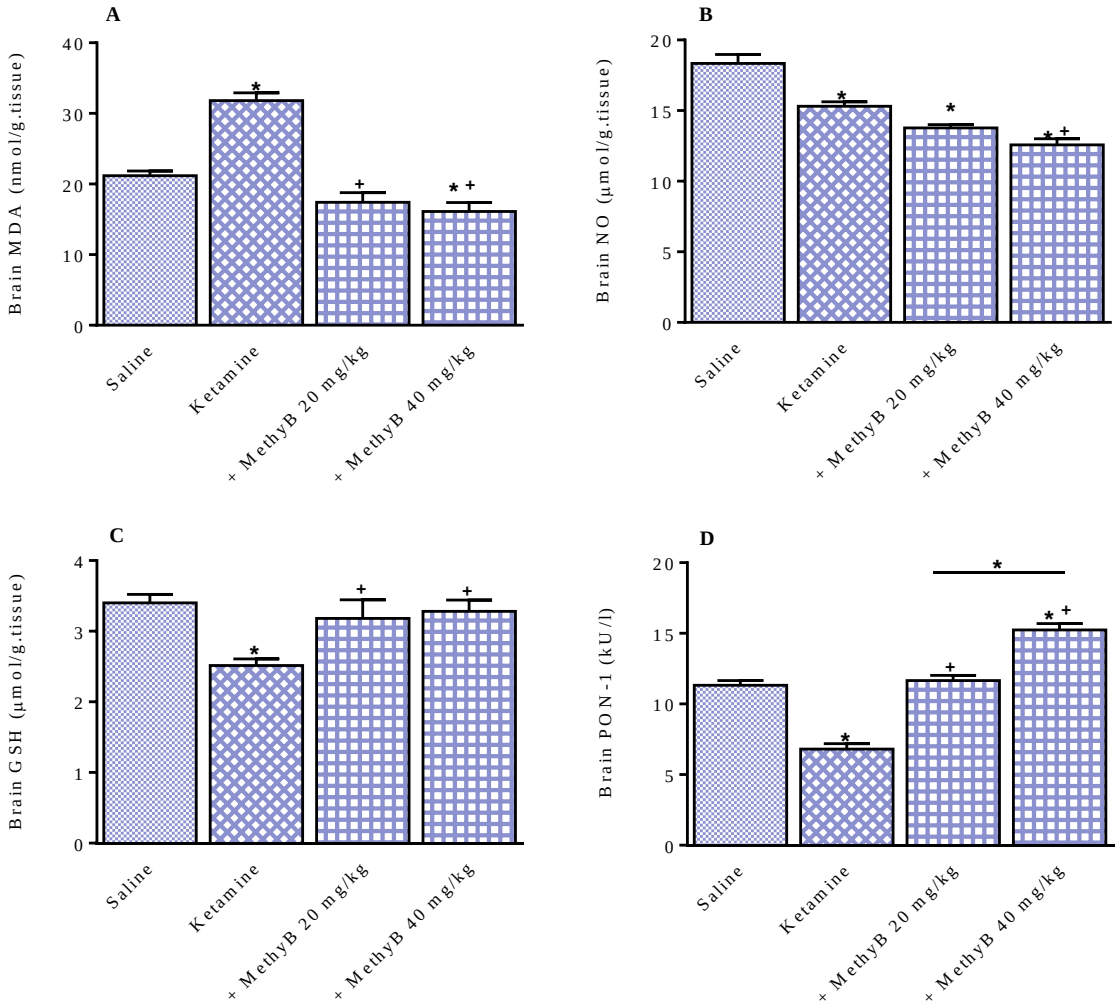

Figure 1 Effect of MethyB on brain malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) and paraoxonase-1 (PON-1) in rats treated with ketamine (35 mg/kg, i.p.). *p<0.05 *vs*. saline and between different groups as indicated in the graph. +p<0.05 *vs*. ketamine control

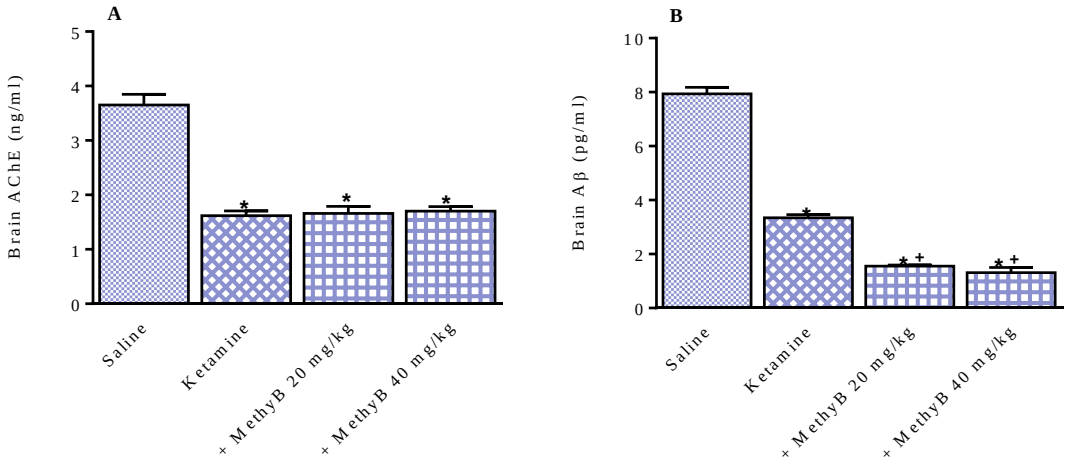

Figure 2 Effect of MethyB on levels of brain acetylcholinesterase (AChE) and amyloid Aβ peptide (Aβ) in rats treated with ketamine (35 mg/kg, i.p.). *p<0.05 *vs*. saline and between different groups as indicated in the graph. +p<0.05 *vs*. ketamine control

Liver reduced glutathione

A significant decrease in the level of GSH by 34.0% was observed in liver of ketamine treated animals compared to their controls (2.77 ± 0.08 *vs*. 4.2 ± 0.1 µmol/g tissue). The ketamine-treated rats receiving MethyB at 20 or 40 mg/kg exhibited significantly higher GSH values by 23.1% and 37.5% in comparison to the ketamine control (3.41 ± 0.09 and 3.81 ± 0.06 *vs*. 2.77 ± 0.08 µmol/g tissue) (Figure 3C).

Liver paraoxonase-1

Following ketamine injection, there was a significant decrease in liver PON-1 activity by 51.7% compared to the saline treated group (14.68 ± 0.72 *vs*. 30.38 ± 1.39 kU/l). MethyB administration at 20 or 40 mg/kg to ketamine-treated rats produced significantly higher levels of PON-1 activity than the ketamine control group (22.27 ± 1.19 and 23.70 ± 0.65 *vs*. 14.68 ± 0.72 kU/l) (Figure 3D).

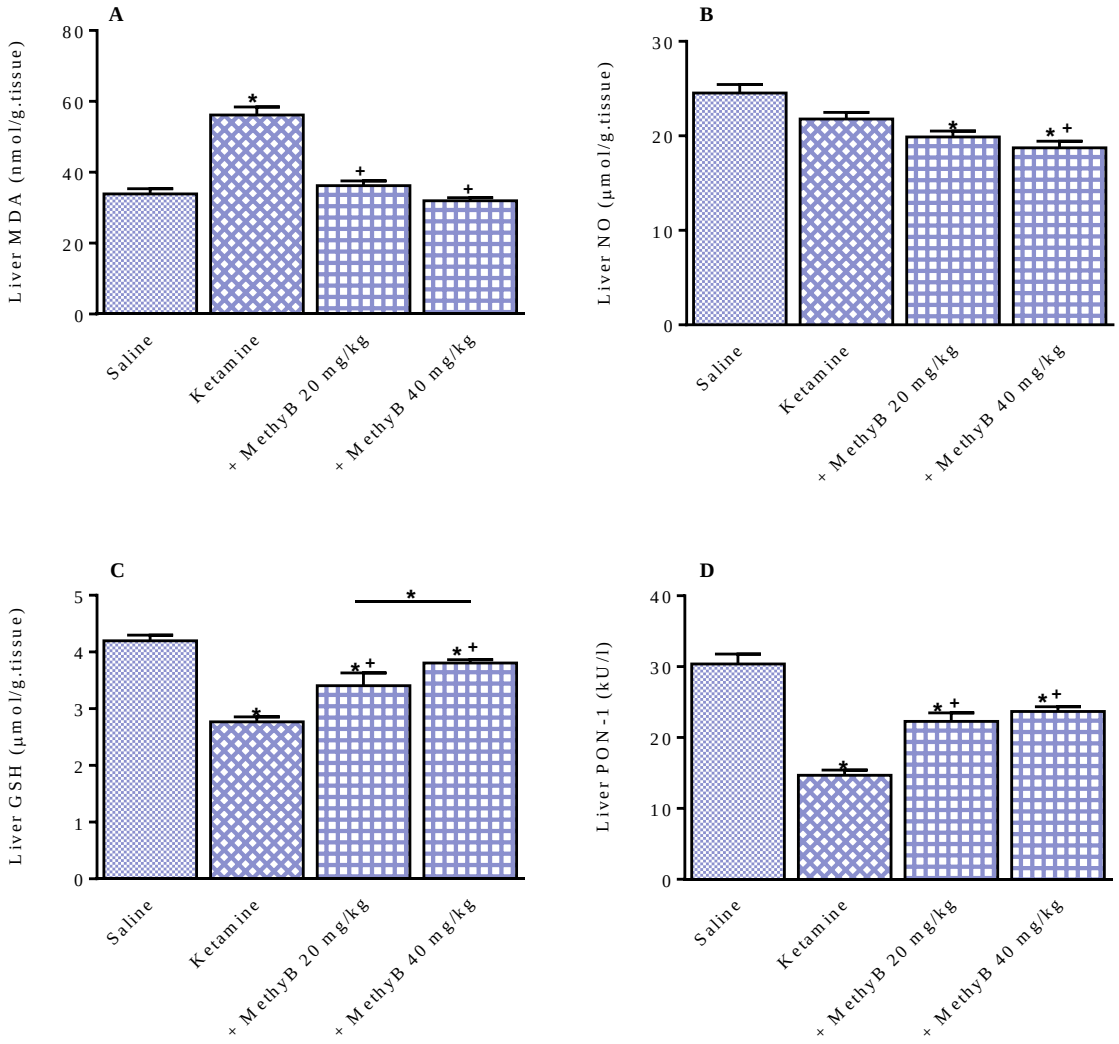

Figure 3 Effect of MethyB on liver malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) and paraoxonase-1 (PON-1) in rats treated with ketamine (35 mg/kg, i.p.). *p<0.05 *vs*. saline and between different groups as indicated in the graph. +p<0.05 *vs*. ketamine control

Effect of MethyB on ketamine–induced brain histologic damage

Cerebral cortex

Sections from saline controls showed the normal histology. The cortical layers had neuronal cells with vesicular nuclei normal, acidophilic cytoplasm with intact blood vessels that had narrow perivascular spaces. Neuroglia cells had well demarcated lightly stained nuclei (Figure 4A). Ketamine-treated group exhibited disturbance and neurodegeneration in the layers of the cortex with

dilated and congested blood vessels. The cortex demonstrated dark shrunken cortical neurons, apoptotic cells with deeply stained pyknotic nuclei and pericellular vacuolations. Many darkly stained glial cells' nuclei were noticed as well (Figure 4B).

In the group treated with ketamine and MethyB at 20 mg/kg, there was moderate improvement in the cortex structure. However, some cortical neurons were still degenerated with pyknotic and darkly stained nuclei. Congested dilated blood vessels were observed. Glial cells appeared normal having lightly or darkly stained nuclei with pericellular space (Figure 4C). Sections from the group treated with ketamine and MethyB at 40 mg/kg showed nearly normal histological structure of the cortex and better neurons which exhibited normal appearance with vesicular nuclei and basophilic cytoplasm, but few cortical neurons were still degenerated with pyknotic and darkly stained nuclei. Glial cells appeared normal and had well demarcated lightly or darkly stained nuclei with slight dilated blood vessels can be observed (Figure 4D).

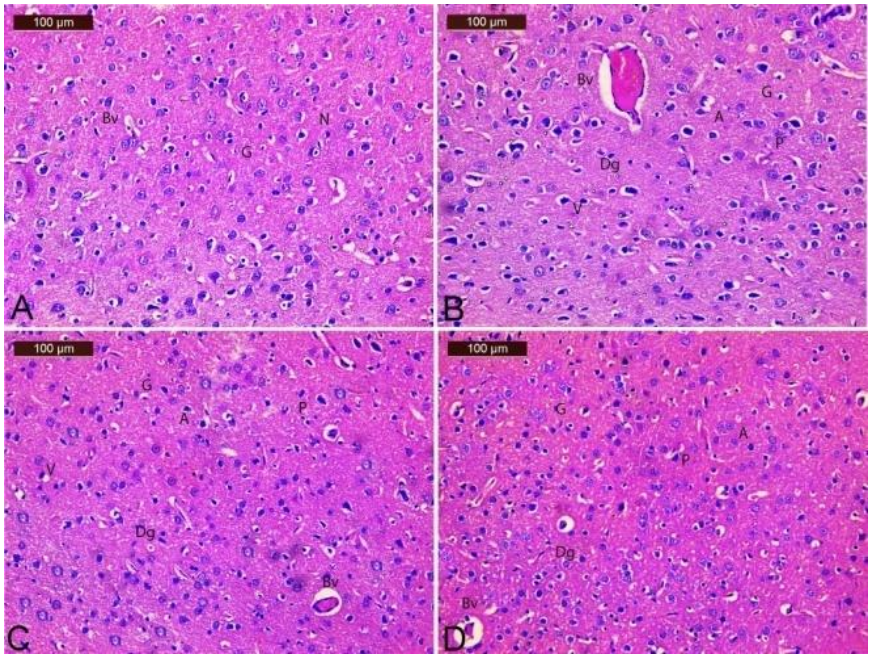

Figure 4 Photomicrographs of Hx & E stained sections of rat cerebral cortex. (A) Saline control group shows well organized cortex layers and normally attached cortical neuronal cells with vesicular nuclei (N), basophilic cytoplasm, blood vessels with narrow perivascular spaces (Bv) and lightly stained nuclei of glial cells (G). (B) Ketamine shows neurodegeneration in cortex with dilated and congested blood vessel (Bv), pyknotic cortical neurons (arrowhead), vacuolated cells (V) with many glial cells that have dark (Dg) or lightly stained nuclei (G). (C) Ketamine and MethyB at 20 mg/kg show moderate improvement with dilated and congested blood vessel (Bv), pyknotic cortical neurons (P), vacuolated cells (V) with many glial cells that have dark (Dg) or lightly (G) stained nuclei. (D) Ketamine and MethyB at 40 mg/kg show remarkable improvement, most cortical neurons (N) nearly normal arrangement of cortical layers. Few cortical neurons still exhibit dark stained nuclei (P), blood vessels with narrow perivascular spaces (BV), glial cells with either lightly (G) or dark (Dg) stained nuclei.

Hippocampus

Sections of saline control group showed C-shaped of hippocampus. This pyramidal layer illustrated the pyramidal cells with prominent nucleoli and vesicular nuclei (Figure 5A). Sections of the hippocampus from the ketamine group demonstrated multiple shrunken degenerated pyramidal cells having dark eosinophilic cytoplasm with pyknotic nuclei and surrounded by pericellular spaces (Figure 5B).

Animals that received ketamine and MethyB at 20 mg/kg showed moderate improvement of the pyramidal layer. Some of the pyramidal neurons displayed vesicular nuclei and prominent nucleoli; others were still having pyknotic nuclei surrounded by pericellular space vacuolation (Figure 5C). In the group treated with ketamine and MethyB at high dose there was considerable protection and normal hippocampus structure as the pyramidal layer showed multiple pyramidal cells and prominent nucleoli and vesicular nuclei with only few pyknotic nuclei being observed (Figure 5D).

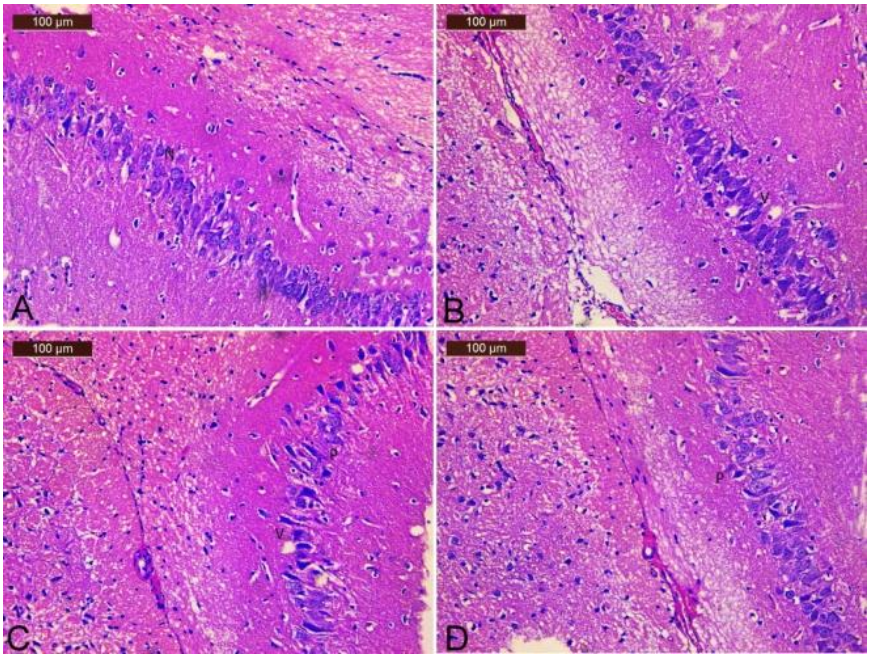

Figure 5 Photomicrographs of Hx & E-stained hippocampus sections. (A) Saline control shows the pyramidal cells with vesicular nuclei and prominent nucleoli (N). (B) Ketamine shows the pyramidal layer with multiple shrunken pyramidal cells which have dark eosinophilic cytoplasm and pyknotic nuclei surrounded (P) by pericellular space vacuolation (V). (C) Ketamine and MethyB at 20 mg/kg show moderate improvement of pyramidal layer with pyknotic nuclei surrounded (P) by pericellular space vacuolation (V). (D) Ketamine and MethyB at 40 mg/kg show many preserved pyramidal cells with vesicular nuclei and prominent nucleoli. Few pyknotic nuclei (P) are observed.

Effect of MethyB on ketamine–induced liver histologic damage

The liver of saline control rats showed normal hepatocytes radiated from central vein and separated by sinusoids with prominent nucleoli and vesicular nuclei (Figure 6A). However, ketamine-treated animals exhibited severe hepatocyte degeneration, markedly dilated central vein, congested with haemolysis blood cells and perivascular inflammatory cells infiltrations with dilated sinusoids filled with haemolytic blood cells and pyknotic nuclei (Figure 6B).

In the animals treated with ketamine and MethyB at 20 mg/kg, the liver showed moderate improvement as normal appearance of central vein, hepatocyte and sinusoids with focally dispersed inflammatory cells aggregation in some areas, with pyknotic nuclei and activation of Kupffer cells (Figure 6C). The liver of rats treated with ketamine and MethyB at 40 mg/kg revealed normal reappearance of most hepatocytes, central vein and sinusoids in wide area. However, some areas still showed mild congested and dilated central vein, and dilated sinusoids (Figure 6D).

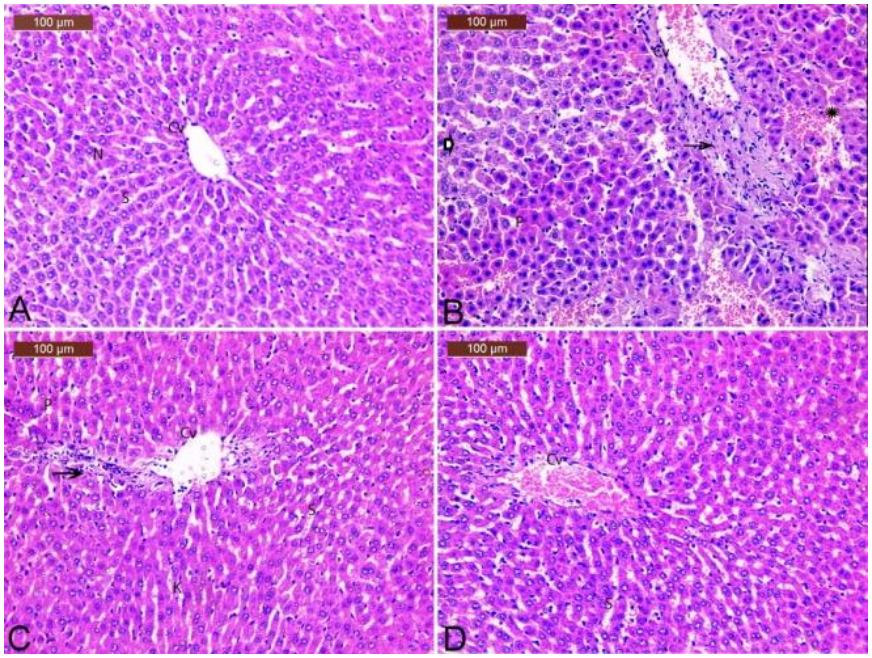

Figure 6 Photomicrographs of Hx & E stained sections of rat liver. (A) Saline control shows normal hepatocytes (H) radiated from central vein (CV) and separated by sinusoid (s). (B) Ketamine shows severe degenerated hepatocytes (arrowhead), marked dilated central vein congested with haemolysed blood cells (CV), perivascular inflammatory cells infiltrations (arrow), and dilated sinusoids filled with haemolysed blood cells (star) and pyknotic nuclei (arrow). (C) Ketamine and MethyB at 20 mg/kg show moderate improvement as revealed by normal appearance of central vein, hepatocytes and sinusoids, focally dispersed inflammatory cells aggregates in some areas (S), pyknotic nuclei (P) and activation of Kupffer cells (K). (D) Ketamine and MethyB at 40 mg/kg show normal reappearance of most hepatocytes, but mild to moderate congested dilated central vein (Cv), and dilated sinusoids (s).

4. DISCUSSION

The present results show that MethyB prevented the ketamine-induced neurotoxicity in the rat brain and that this neuroprotective effect was associated with a decrease in the level of oxidative stress. This finding provides further support to previous studies that indicated a neuroprotective effect for MethyB in toxic and inflammatory models of brain damage (Bardakci et al., 2006; Sontag et al., 2012; Abdel-Salam et al., 2014; Abdel-Salam et al., 2016; Dibaj et al., 2010). In this study, following a single i.p. ketamine injection, there was a significant increase in brain oxidative stress as indicated by the increase in lipid peroxidation assessed by measuring MDA (Gutteridge, 1995).

This was accompanied by a significant decrease of the antioxidant and free radical scavenger molecule GSH, suggesting that ketamine induces the release of reactive oxidant species with subsequent consumption of the antioxidant GSH. Our results also indicated that ketamine caused a significant decrease in paraoxonase-1 (PON-1) activity which is in agreement with previous reports (Abdel-Salam et al., 2015b; Abdel-Salam et al., 2018; Abdel-Salam et al., 2021). Paraoxonase-1 is involved in xenobiotic metabolism (Primo-Parmo et al., 1996) and in protection against oxidative and inflammatory events (Mackness and Mackness, 2015).

The activity of PON-1 decreases in serum from patients with liver disease (Camps et al., 2009) and in a number of neurological disorders (Menini and Gugliucci, 2014; Abdel-Salam et al., 2015a). This decrease in PON-1 activity is thought to reflect oxidative inactivation of the enzyme by the increase in reactive oxygen species (Aviram, 1999). In addition, ketamine induced neuronal degeneration indicated by the presence of dark shrunken cortical neurons with deeply stained pyknotic nuclei and shrunken degenerated pyramidal cells having dark eosinophilic cytoplasm in hippocampus. These findings add to previous studies that showed the presence of increased oxidative stress, shrunken apoptotic neurons, perineuronal vacuolations and increased caspase-3 immunoreactivity in the brain of rats after treatment with ketamine (Abdel-Salam et al., 2015b; Abdel-Salam et al., 2018).

In the study by Zou et al., (2009) degenerating neurons in brain of neonatal rats were observed after repeated administration of ketamine at 20 mg/kg. A study in mice found that i.p. injection of ketamine at 50 or 100 mg/kg caused cytoplasmic vacuolation in pyramidal neurons (Chung and Yoon, 2008). A neurotoxic effect for ketamine was also reported in human neuronal cells in vitro,

which is caused by increased production of reactive oxygen species that induced cytochrome C release from mitochondria and mitochondrial apoptosis (Braun et al., 2010; Bosnjak et al., 2012).

The mechanism by which ketamine causes neuronal injury may thus involve oxidative stress. In support of this notion is the finding that treatment with the glutathione precursor *N*-Acetylcysteine was able to ameliorate the brain neuronal damage that followed ketamine injection in the rat (Abdel-Salam et al., 2018). Our results also demonstrated a significant decrease in brain AChE levels by ketamine treatment which suggested that ketamine may modulate cholinergic neurotransmission. In microdialysis experiments, Kikuchi et al., (1997) reported marked increase in acetylcholine (ACh) release from the rat frontal cortex after i.p. injection of ketamine 25, 50 and 100 mg/kg.

A similar finding was also reported by the studies of Kim et al., (1999) and Nelson et al., (2002) in which the acute administration of ketamine was found to cause a significant rise in cortical release of acetylcholine compared to basal levels. In rabbits, ketamine induced a significant cerebral vasodilatation that was blocked by the cholinergic antagonist scopolamine (Reicher et al., 1987). The increase in cortical acetylcholine has been postulated to account for the neurotoxic action of NMDA antagonists (Nelson et al., 2002). These cholinergic effects may be also implicated in the ketamine-induced hallucinogenic and psychotomimetic properties. Ketamine induces perceptual distortion of time and place, hallucinations and dissociative effects (De-Luca et al., 2012).

In the present study, the effect of ketamine on brain Aβ content was quantitatively measured using ELISA. An intriguing observation was that the acute administration of ketamine caused significant decrement in brain Aβ-peptide content. Amyloid β-protein (Aβ) is produced from the amyloid β-protein precursor through sequential cleavage by β- and γ-secretases. The deposition of extracellular amyloid plaques composed mainly of the amyloid-β (Aβ) peptide is an important pathological hallmark of Alzheimer's disease (Serrano-Pozo et al., 2011).

These Aβ aggregates are largely thought to be the initiating event in the neurodegenerative process in Alzheimer's disease (Rajmohan and Reddy, 2017). The mechanism by which a single dose of ketamine induces a significant decrease in brain Aβ-peptide is yet to be established. Liver injury evidenced by increased serum activities of liver enzymes has been reported in abusers of ketamine (Wong et al., 2014).

In the present study, similar to the effects of ketamine in brain, there was a significant increase in MDA concentrations along with decreased GSH in liver tissue. In addition, a significant decrease in PON1 activity in liver tissue was observed after treatment with ketamine. The liver of ketamine-treated rats exhibited severe degeneration of hepatocytes, perivascular inflammatory cells infiltrations and dilated sinusoids filled with haemolytic blood cells. These findings are in agreement with a previous study in which ketamine given i.p. at 30 mg/kg in rats caused increased liver oxidative stress, necrosis of hepatocytes, congestion of sinusoids as well as positive caspase-3 immunostaining (Abdel-Salam et al., 2015b).

Our present study provided the first evidence that treatment with MethyB was able to alleviate the neuronal and hepatic toxic effects of ketamine. MethyB significantly decreased lipid peroxidation, increased GSH and PON-1 activity in brain and liver tissue and afforded protection against histologic brain and liver injury caused by ketamine. These findings add to previous reports of a protective effect for MethyB in experimental models of neurodegenerative disease (Bardakci et al., 2006; Sontag et al., 2012; Abdel-Salam et al., 2014).

Methylene blue was also shown to decrease brain edema, apoptotic and degenerating neurons and to prevent liver damage in the toxicant e.g., toluene- or malathion-induced brain and liver pathologies (Abdel-Salam et al., 2016a; Abdel-Salam et al., 2016b). MethyB in addition decreased serum oxidative stress, number of dark shrunken, apoptotic neurons, and caspase-3 activation in brain as well as vacuolar degeneration of hepatocytes in rats with lipopolysacchardie-induced systemic inflammation (Abdel-Salam et al., 2015a).

MethyB is an inhibitor of guanylate cyclase and nitric oxide synthases and thus inhibits the production of nitric oxide (Mayer et al., 1993; Volke et al., 1999) which provides explanation for the observed decrease in brain nitric oxide by MethyB in the present study. MethyB also interacts with the cholinergic system. Significant inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase by MethyB or its metabolite azure B in vitro has been reported (Pfaffendorf et al., 1997; Petzer et al., 2014). In the present study, however, the administration of MethyB did not result in further decrease in brain AChE beyond that caused by ketamine.

The present findings in addition indicated that the administration of MethyB was associated with a further decrease in Aβ-peptide content in brain of ketamine-treated rats. In a genetic mouse model of Alzheimer disease, MethyB was reported to reduce Aβ levels and improve learning and memory deficits (Medina et al., 2011). Under physiological conditions, MethyB undergoes a catalytic redox cycle between its oxidized blue cation and the reduced colorless leucoMethyB forms, which may block production

by mitochondria of reactive oxygen metabolites and hence prevents further mitochondrial damage by these oxidant species (Atamna and Kumar, 2010).

Moreover, MethyB has been reported to exert antioxidative effects (Salaris et al., 1991), enhance brain metabolism, increasing brain glucose uptake and cerebral blood flow (Lin et al., 2012). These actions of MethyB besides a decrease in level of oxidative stress could have accounted for the protective effects of the dye in the present study.

5. CONCLUSIONS

In summary, MethyB demonstrated a protective action against brain neurodegeneration and liver damage induced by ketamine in the rat. It is suggested that this action of MethyB is caused by lowered oxidative stress levels. The study provides evidence that MethyB may be of value in treatment of toxicity caused by ketamine abuse.

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Authors' Contributions

OMEAS: Study conception and design and data analysis; SAA: Experimental studies and data analysis; ERY: Biochemical analysis; OEA: Histopathological studies and interpretation; OMEAS, SAA, OEA and ERY: Manuscript preparation, revision and final approval of the version to be published.

Consent for publication

Not applicable

Informed consent

Not applicable.

Ethical approval

The animal studies were done according to regulations of the Institute Ethics Committee and Guide for Care and Use of Laboratory Animals of US National Institutes of Health (Publication No. 85-23, revised 1996).

Conflicts of interests

The authors declare that there are no conflicts of interests.

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Data and materials availability

All data associated with this study are present in the paper.

REFERENCES AND NOTES

1. Abdel-Salam OME, El-Shamarka MES, Omara EA. Brain oxidative stress and neurodegeneration in the ketamine model of schizophrenia during antipsychotic treatment: effects of *N*-acetylcysteine treatment. Reactive Oxygen Species 2018; 6(16):253–266.
2. Abdel-Salam OME, Omara EA, Youness ER, Khadrawy YA, Mohammed NA, Sleem AA. Rotenone-induced nigrostriatal toxicity is reduced by methylene blue. J Neurorestoratol 2014; 2:65–80.
3. Abdel-Salam OME, Youness ER, Esmail RSE, Mohammed NA, Khadrawy YA, Sleem AA, Abdulaziz AM. Methylene blue as a novel neuroprotectant in acute malathion intoxication. Reactive Oxygen Species 2016a; 1(2):165–177.
4. Abdel-Salam OME, Youness ER, Mohammed NA, Abu-Elhamed WA. Nuclear factor-kappa B and other oxidative stress biomarkers in serum of autistic children. Open J Mol Integr Physiol 2015a; 5:18–27.
5. Abdel-Salam OME, Youness ER, Mohammed NA, Omara EA, Sleem AA. Effect of ketamine on oxidative stress following lipopolysaccharide administration. Comp Clin Pathol 2015b; 2 4:53–63. doi: 10.1007/s00580-013-1854-x

6. Abdel-Salam OME, Youness ER, Morsy FA, Yassen NN, Mohammed NA, Sleem AA. Methylene blue protects against toluene induced brain damage. Involvement of nitric oxide, NF-κB, and caspase-3. Reactive Oxygen Species 2016b; 2(5):371–387.
7. Abdel-Salam OME, Youness ER, Sleem AA, Omara EA. Oxidative stress and neuronal injury after cannabis and ketamine administration. Wseas Trans Biol Biomed 2021; 18: 126-135. doi: 10.37394/23208.2021.18.15
8. Ahern TL, Herring AA, Anderson ES, Madia VA, Fahimi J, Frazee B. The first 500: Initial experience with widespread use of low dose ketamine for acute pain management in the ED. Am J Emerg Med 2015; 33(2):197–201.
9. Archer S. Measurement of nitric oxide in biological models. FASEB J 1993; 7(2):349–60.
10. Atamna H, Kumar R. Protective role of methylene blue in Alzheimer's disease via mitochondria and cytochrome c oxidase. J Alzheimers Dis 2010; 20 Suppl 2:S439-52. doi: 10.3233/JAD-2010-100414
11. Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, Newton RS, Du BL. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. Free Radic Biol Med 1999; 26 (7-8):892-904.
12. Bardakci H, Kaplan S, Karadeniz U, Ozer C, Bardakci Y, Ozogul C, Birincioglu CL, Cobanoglu A. Methylene blue decreases ischemia-reperfusion (I/R)-induced spinal cord injury: An in vivo study in an I/R rabbit model. Eur Surg Res 2006; 38(5):482–488.
13. Beaudoin FL, Lin C, Guan W, Merchant RC. Low-dose ketamine improves pain relief in patients receiving intravenous opioids for acute pain in the emergency department: Results of a randomized, double-blind, clinical trial. Acad Emerg Med 2014; 21:1193–1202.
14. Bosnjak ZJ, Yan Y, Canfield S, Muravyeva MY, Kikuchi C, Wells C, Corbett JA, Bai X. Ketamine induces toxicity in human neurons differentiated from embryonic stem cells via mitochondrial apoptosis pathway. Curr Drug Saf 2012; 7(2):106–119.
15. Braun S, Gaza N, Werdehausen R, Hermanns H, Bauer I, Durieux ME, Hollmann MW, Stevens MF. Ketamine induces apoptosis via the mitochondrial pathway in human lymphocytes and neuronal cells. Br J Anaesth 2010; 105(3):347–54.
16. Camps J, Marsillach J, Joven J. Measurement of serum paraoxonase-1 activity in the evaluation of liver function. World J Gastroenterol 2009; 15:1929–1933.
17. Chung EY, Yoon JR. Ketamine potentiates neurotoxicity in obese mice. Korean J Anesthesiol 2008; 55(4):473-478. doi: 10.4097/kjae.2008.55.4.473
18. De-Luca MT, Meringolo M, Spagnolo PA, Badiani A. The role of setting for ketamine abuse: Clinical and preclinical evidence. Rev Neurosci 2012; 23(5-6):769–780.
19. Dibaj P, Zschüntzsch J, Steffens H, Scheffel J, Göricke B, Weishaupt JH, Le-Meur K, Kirchhoff F, Hanisch UK, Schomburg ED, Neusch C. Influence of methylene blue on microglia-induced inflammation and motor neuron degeneration in the SOD1G93A model for ALS. PLoS One 2010; 7(8):e43963.
20. Drury RVA, Walligton EA. Carleton's Histological Technique, 5th edition. Oxford University Press, New York 1980; 206.
21. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82(1):70–77.
22. Gutteridge JMC. Lipid peroxidation and anti-oxidants as biomarkers of tissue damage. Clin Chem 1995; 41:1819-28.
23. Haagen L, Brock A. A new automated method for phenotyping arylesterase (EC 3.1.1.2) based upon inhibition of enzymatic hydrolysis of 4-nitrophenyl acetate by phenyl acetate. Eur J Clin Chem Clin Biochem 1992; 30(7):391-395.
24. Halliwell B. Oxidative stress and neurodegeneration: Where are we now? J Neurochemistry 2006; 97:1634–1658.
25. Halliwell B. The wanderings of a free radical. Free Radic Biol Med 2009; 46(5):531–42.
26. Kikuchi T, Wang Y, Shinbori H, Sato L, Okumura F. Effects of ketamine and pentobarbitone on acetylcholine release from the rat frontal cortex in vivo. Br J Anaesth 1997; 79:128–130.
27. Kim SH, Price MT, Olney JW, Farber NB. Excessive cerebrocortical release of acetylcholine induced by NMDA antagonists is reduced by GABAergic and α2-adrenergic agonists. Mol Psychiatry 1999; 4(4):344–352.
28. Klein JA, Ackerman SL. Oxidative stress, cell cycle, and neurodegeneration. J Clin Investig 2003; 111(6):785-793.
29. Lankenau SE, Clatts MC. Drug injection practices among high-risk youths: The first shot of ketamine. J Urban Health 2004; 81(2):232–248.
30. Lin AL, Poteet E, Du F, Gourav RC, Liu R, Wen Y, Bresnen A, Huang S, Fox PT, Yang SH, Duong TQ. Methylene blue as a cerebral metabolic and hemodynamic enhancer. PLoS One 2012; 7(10):e46585. doi: 10.1371/journal.pone.0046585
31. Mackness M, Mackness B. Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles. Gene 2015; 567(1):12–21. doi: 10.1016/j.gene.2015.04.088
32. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. Biochem Pharmacol 1993; 45(2):367–374.
33. Medina DX, Caccamo A, Oddo S. Methylene blue reduces abeta levels and rescues early cognitive deficit by increasing proteasome activity. Brain Pathol 2011; 21(2):140–9. doi: 10.1111/j.1750-3639.2010.00430.x

34. Menini T, Gugliucci A. Paraoxonase 1 in neurological disorders. Redox Rep 2014; 19(2):49–58.
35. Mion G, Villevieille T. Ketamine pharmacology: An update (pharmacodynamics and molecular aspects, recent findings). CNS Neurosci Ther 2013; 19(6):370–380.
36. Nair V, Turner GA. The thiobarbituric acid test for lipid peroxidation: Structure of the adduct with malondialdehyde. Lipids 1984; 19(10):804-805.
37. Neill JC, Barnes S, Cook S, Grayson B, Idris NF, Mc-Lean SL, Snigdha S, Rajagopal L, Harte MK. Animal models of cognitive dysfunction and negative symptoms of schizophrenia: Focus on NMDA receptor antagonism. Pharmacol Ther 2010; 128(3):419–432.
38. Nelson CK, Burk JA, Bruno JP, Sarter M. Effects of acute and repeated systemic administration of ketamine on prefrontal acetylcholine release and sustained attention performance in rats. Psychopharmacology (Berl) 2002; 161(2):168–179.
39. Niesters M, Martini C, Dahan A. Ketamine for chronic pain: Risks and benefits. Br J Clin Pharmacol 2014; 77:357–367.
40. Petzer A, Harvey BH, Petzer JP. The interactions of azure B, a metabolite of methylene blue, with acetylcholinesterase and butyrylcholinesterase. Toxicol Appl Pharmacol 2014; 274(3):488–93. doi: 10.1016/j.taap.2013.10.014
41. Pfaffendorf M, Bruning TA, Batnik HD, Zwieten PA. The interaction between methylene blue and the cholinergic system. Br J Pharmacol 1997; 122(1):95–8. doi: 10.1038/sj.bjp.0701355
42. Pomarol-Clotet E, Honey GD, Murray GK, Corlett PR, Absalom AR, Lee M, McKenna PJ, Bullmore ET, Fletcher PC. Psychological effects of ketamine in healthy volunteers. Phenomenological study. Br J Psychiatry 2006; 189:173–179.
43. Primo-Parmo SL, Sorenson RC, Teiber L, La-Du BN. The human serum paraoxonase/arylesterase gene (PON1) is one member of multigene family. Genomics 1996; 33(3):498–507.
44. Rajmohan R, Reddy PH. Amyloid beta and phosphorylated tau accumulations cause abnormalities at synapses of Alzheimer's disease neurons. J Alzheimers Dis 2017; 57:975-999.
45. Reicher D, Bhalla P, Rubinstein EH. Cholinergic cerebral vasodilator effect of ketamine in rabbits. Stroke 1987; 18:445-449.
46. Salaris SC, Babbs CF, Voorhees WD. Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. A potential new drug for the attenuation of ischemia/reperfusion injury. Biochem Pharmacol 1991; 42:499–506.
47. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med 2011; 1(1):a006189.
48. Sies H. Oxidative stress: Oxidants and antioxidants. Exp Physiol 1997; 82(2):291-295.
49. Sontag EM, Lotz GP, Agrawal N, Tran A, Aron R, Yang G, Necula M, Lau A, Finkbeiner S, Glabe C, Marsh JL, Muchowski PJ, Thompson LM. Methylene blue modulates huntingtin aggregation intermediates and is protective in Huntington's disease models. J Neurosci 2012; 32(32):11109–19.
50. Talley-Watts L, Long JA, Chemello J, Van-Koughnet S, Fernandez A, Huang S, Shen Q, Duong TQ. Methylene blue is neuroprotective against mild traumatic brain injury. J Neurotrauma 2014; 31(11):1063–1071.
51. Trujillo KA, Smith ML, Sullivan B, Heller CY, Garcia C, Bates M. The neurobehavioral pharmacology of ketamine: implications for drug abuse, addiction, and psychiatric disorders. ILAR J 2011; 52(3):366-378.
52. Volke V, Wegener G, Vasar E, Rosenberg R. Methylene blue inhibits hippocampal nitric oxide synthase activity in vivo. Brain Res 1999; 826(2):303–305.
53. Wilson CA, Koenig JI. Social interaction and social withdrawal in rodents as readouts for investigating the negative symptoms of schizophrenia. Eur Neuropsychopharmacol 2014; 24(5):759–773.
54. Wolff K. Ketamine. In: Verster JC (editor), Drug abuse and addiction in medical illness: Causes, consequences and treatment. Springer, Heidelberg 2012; 201–211.
55. Wong GL, Tam YH, Ng CF, Chan AW, Choi PC, Chu WC, Lai PB, Chan HL, Wong VW. Liver injury is common among chronic abuses of ketamine. Clin Gastroenterol Hepatol 2014; 12(10):1759–1762.
56. Zou X, Patterson TA, Sadovova N, Twaddle NC, Doerge DR, Zhang X, Fu X, Hanig JP, Paule MG, Slikker W, Wang C. Potential neurotoxicity of ketamine in the developing rat brain. Toxicol Sci 2009; 108(1):149-58. doi: 10.1093/toxsci/kfn270